ANTI-VEGFR2 NANOGELS AS A DELIVERY SYSTEM OF THERAPEUTIC AGENTS TO THE BRAIN TUMOR CELLS

Korchagina A.A.[1], Nukolova N.V.[1,2,3], Shein S.A.[1,3], Abakumova T.O.[1], Chekhonin V.P.[1,2]

[1]*Department of Fundamental and Applied Neurobiology, The Serbsky State Scientific Center for Social and Forensic Psychiatry, 119991, Kropotkinsky Per. 23, Moscow, Russia*

[2]*Department of Medicinal Nanobiotechnologies, Pirogov Russian National Research Medical University, 117997, Ostrovityanova Str. 1, Moscow, Russia*

[3]*Department of Chemistry, Lomonosov Moscow State University, Leninskie Gory, Moscow, 119992, Russia*

Abstract

Conventional approaches in case of primary malignant brain tumors are still low effective in inhibition of tumor progression. Targeted drug delivery using nanocarriers conjugated with monoclonal antibody is an attractive anti-tumor strategy that may significantly improve efficacy of conventional chemotherapeutics delivery to the tumor. Our purpose was to investigate the ability of synthesized nanogels directed against vascular endothelial growth factor receptor II (VEGFR2), a surface receptor widely expressed on activated endothelial cells as well as at sites of tumor angiogenesis. Anti-VEGFR2 nanogels have shown specific binding not only to the rat glioma C6 cells but also to human U-87 MG cells versus unspecific nanogels and nanogels conjugated with IgG. The fluorescence confocal microscopy has revealed that unspecific nanogels and IgG-nanogels have low fluorescent intensity and their localisations within cells are strongly different. These results suggest that targeted anti-VEGFR2 nanogels should be a promising system for selective delivery of chemotherapeutic agents to the VEGFR2 expressing brain tumors.

Keywords: Brain tumor, glioma, targeted delivery, anti-VEGFR2 antibody, anti-VEGFR2 nanogels

INTRODUCTION

Conventional and combination therapies are still low effective in tumors of the central nervous system. A primary cause is that the drugs poorly penetrate into tumor cells and have broad range of adverse side effects. The problem is especially presented in neurooncology because intracranial neoplasms are accompanied by high intratumoral pressure and vasogenic edema. Moreover, high-grade gliomas (HGG) are the most rapidly progressing and aggressive tumors among primary brain tumors that are characterized by a high growth rate, intense angiogenesis, invasive growth, high recurrence rates after surgery and high resistance to chemotherapy and radiation [1]. Median survival remains less than 15 months in case of glioblastoma multiforme. Accordingly, improving the selectivity of cytostatic drugs is a main problem of antitumor chemotherapy.

Targeted delivery systems are an attractive strategy, on the one hand, to overcome limitations of chemotherapy, on the other hand, to improve efficiency in treatment of brain tumors. Controlled drug biodistribution at the cell level will make it possible to increase the drug concentration within the tumor and to reduce the systemic toxicities of drugs with low therapeutic index. Nowadays, humanized monoclonal antibodies to various proteins are generated and widely used in medicine [2]. In addition, liposome-encapsulated drug forms have already found applications in clinical practice. However, targeted systems are still at the stage of laboratory and preclinical investigation [3][4].

Endothelial cells play a crucial role in tumor microenvironment by directly interacting with the tumor cells. Moreover, endothelial cells produce cytokines such as VEGF, PDGF and PIGF that regulate tumor angiogenesis [5], cell survival, proliferation and so forth. Activated endothelial cells express VEGFR2 and its expression increased in angiogenesis sites including tumor microvasculature. Furthermore, tumor-produced

VEGF binding with VEGFR2 promote pro-survival signaling by tumor cells and play an important role in tumor resistance to the conventional and combination therapies [6]. Thus, VEGFR2 represent an attractive molecular target for site-directed drug delivery. It should be noted that VEGFR2 overexpression has been shown not only on activated endothelial cells but also on the cell surface of human glioblastoma multiforme stem-like cells [7]. Several studies have shown that employing VEGFR2 targeted liposomes were able to improve cellular uptake and enhance intratumoral accumulation [8][9]. However, most of these studies are focused on liposomes and there is no evidence in using nanogels as a nanocarrier for drug delivery of therapeutics to the brain tumors. The purpose of this study was to investigate the ability of anti-VEGFR2 targeted nanogels to accumulate in brain tumor cells *in vitro.*

1. MATERIALS AND METHODS

1.1. Cell Lines

C6 rat glioma cells and U87 MG (Human glioblastoma-astrocytoma, epithelial-like cell line) were obtained from the ATCC. C6 and U-87 MG cells were grown as monolayer cultures in RPMI 1640 and DMEM medium, respectively, supplemented with 5% (v/v) FBS, 1 mM glutamine, penicillin (100 units/ml) and streptomycin (0,1 mg/ml) under 5% CO2 at 37 °C. Cells were trypsinized using 0,1% trypsin in Ca2+-free, Mg2+-free PBS and 0,025% EDTA after 70-80% confluence.

1.2. Antibodies against VEGFR2

Monoclonal antibodies against I-III extracellular Ig-like domains of VEGFR2 were obtained as described earlier[10]. Briefly, BALB/c mice were immunized with recombinant human I-III extracellular Ig-like domains of VEGFR2. Splenocytes were fused with cultured SP2/0-Ag14 myeloma cells using PEG/DMSO. Hybridomas were tested and selected by ELISA, western blotting, and immunocytochemistry. The affinity constant of the resulting antibody was estimated according to *Beatty et al.* [11]

Monoclonal antibody against VEGFR2 was isolated from BALB/c mice ascites via affine chromatography on agarose- linked protein A, then anti-VEGFR2 antibody was dialyzed against PBS (pH 7,4) and concentrated to 5-8 mg/ml using 3-kDa centrifugal filter units (Millipore, US)

1.3. Conjugation of anti-VEGFR2 to nanogel-PEG-MAL

Poly(ethylene glycol)$_{170}$-b-poly(methacrylic acid)$_{180}$ nanogels (PEG-b-PMAA) were prepared as described earlier [12] using 1,2 ethylene diamine for cross-linking.

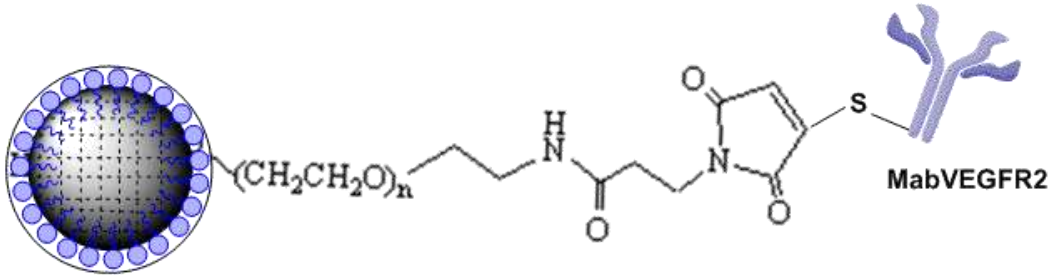

Fig. 1. Scheme of prepared anti-VEGFR2 nanogels. Nanogel conjugated with anti-VEGFR2 antibody through MAL-PEG-NH_2.

Conjugation of anti-VEGFR2 antibody to nanogel-PEG-MAL was performed via thiol group as described previously [13].The antibody added to Traut's reagent for 45 min at r.t with ratio of 1:10. Excess of Traut's reagent was moved and purified antibodies were incubated with MAL-PEG-NH_2 (overnight, 4°C). Then activated nanogels were added to antiVEGFR2-PEG-NH_2 (PBS, pH 7,4) and stirred for 4 h at r.t. Conjugated

anti-VEGFR2 nanogel (Fig. 1) was purified using filtration on Amicon filters (MWCO 30 kDa, Millipore) at 3000 rpm for 10 min. As a control, IgG was conjugated to nanogel in the same way.

In order to evaluate whether the anti-VEGFR2 antibody has reactivity after conjugation with nanogels, ELISA was performed.

1.4. Immunocytochemistry

Cells of the C6 and U-87 MG were fixed with 4% paraformaldehyde in PBS (pH 7,4) at r.t. for 30 min. PBS with 0,2% Tween-40 was used as a working buffer. To reduce nonspecific absorption cells were treated with 2% normal goat serum. The anti-VEGFR2 antibody at 2-10 μg/ml was used. As a negative control, nonspecific mouse immunoglobulins (IgG) purified from a Sp2/0-Ag14 ascites fluid were used at an equivalent concentration. Cells were incubated with primary antibodies for 3 h. at r.t. or at 4°C for 12 h. An Alexa Fluor 488 goat anti-mouse immunoglobulin antibody (Invitrogen, US) was used as a secondary antibody at a 1:1000 dilution. Cell nuclei were stained with DAPI (Invitrogen, US).

1.5. Confocal microscopy

Cells (50 000 cells per chamber) were grown in corresponding media for 2 days. FITC-labeled nanogels were added to the cells at 35 pmol/ml and incubated for 1 h. Nonmodified nanogels and nanogels conjugated with nonspecific IgG were used as a control.

2. RESULTS

2.1. Analysis of Targeting Antibodies

We used an anti-mouse VEGFR2 monoclonal antibody with high affinity against native VEGFR2 that was obtained previously [10]. In order to check whether VEGFR2 is promising molecular target in the case of C6 glioma and whether produced monoclonal antibody is suitable as a targeting moiety. It is widely known that C6 cells expressed VEGFR2 [14][15]. The rat C6 cell line and U-87 MG cell lines were chosen for the establishment of an *in vitro* model of brain tumor nanogel targeting. To ensure that these cell lines expressed VEGFR2 on their surface, ICH was used. A fluorescence analysis showed that anti-VEGFR2 antibody intensely stained both rat C6 and human U-87 MG cells (Fig. 2). Based on these data, all experiments were carried out with these cell lines.

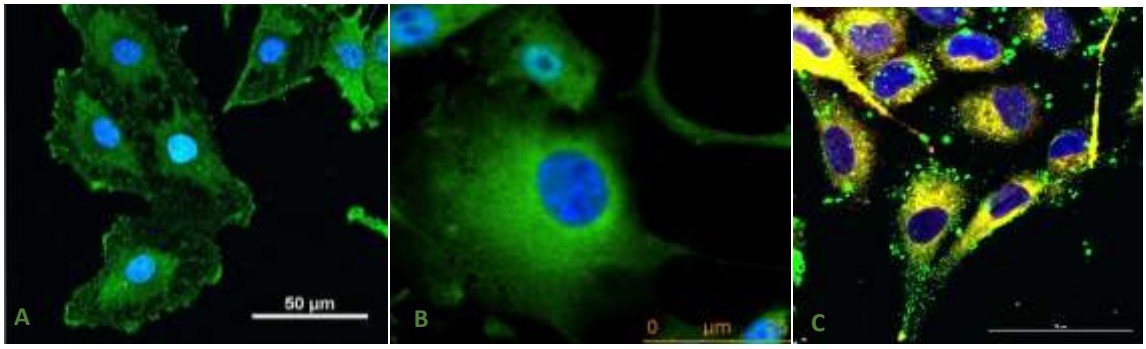

Fig. 2. Immunofluorescent analysis of VEGFR2 in fixed and live cell cultures. Green fluorescence reveals anti-VEGFR2, Yellow fluorescence, Dil. Cell nuclei were stained with DAPI. A. Fixed C6 cell line (confocal microscopy); B. Fixed U-87 MG cells; D. Live C6 cells (confocal microscopy).

PEG-b-PMAA negatively charged nanogels were chosen as a nanocarrier in our work. The anti-VEGFR2 monoclonal antibody and nonspecific antibodies (IgG) were attached as targeting molecules by binding their sulfhydryl groups to maleimide groups on the nanogels surface. Antibodies were incubated with a thiolating agent (2-iminothiolane), which reacts with free primary amino groups (e.g., the ε amino groups of lysine

residues) of a protein to add an SH-containing group. Thiolated antibodies were immediately conjugated with fresh made nanogels because maleimide groups are prone to hydrolysis in solution. It is important to note that the 2-iminothiolane amount affected the immunochemical activity of antibodies as measured by ELISA. We estimated the immunochemical activity for antibodies thiolated using a 5-, 10-, 15-, or 20-fold excess of 2-iminothiolane. A 10-fold excess proved to be optimal, allowing the antibodies to save up to 90% of their initial immunochemical activity.

The resulting targeted nanogels are particles with a diameter 90±10 nm and a polydispersity index of 0,1±0,05. The amount of conjugated antibodies was evaluated by micro BCA assay and was 100-150 μg per mg of nanogel.

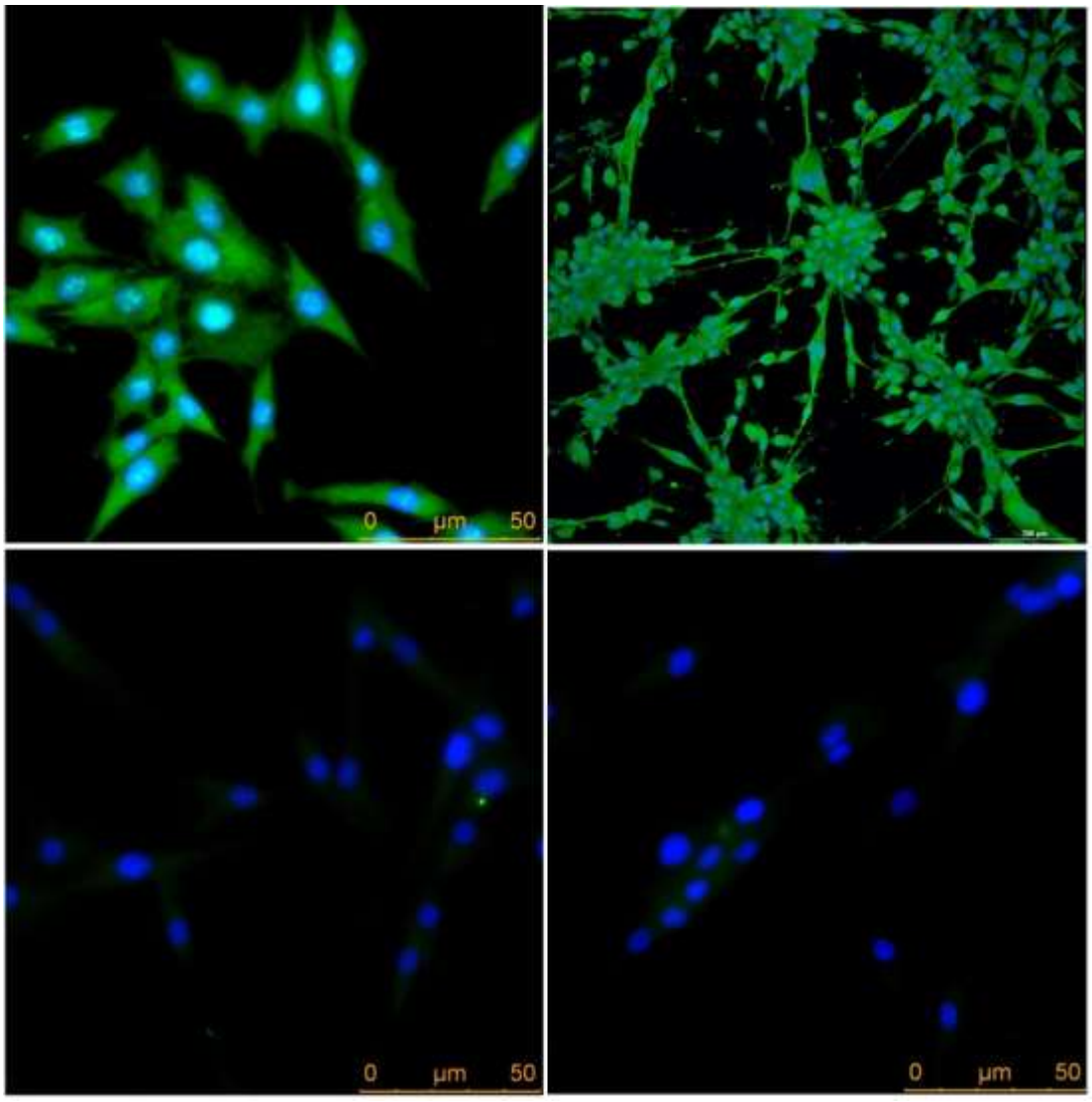

Fig. 3. Fluorescence analysis of nanogel accumulation on C6 glioma cells and U-87 MG. Cell visualization after treatment with (A, B) anti-VEGFR2 nanogels (green) in contrast to (C) nanogels without conjugated antibodies or (D) nanogels conjugated with nonspecific immunoglobulins. nanogels (green), nuclei were stained DAPI (blue).

One of the main problems of antibody conjugation is that it may lose affinity for their molecular target when the groups responsible for their binding are modified. In addition, some antibody molecules may be shielded by PEG chains or other antibody molecules conjugated to the surface of the same nanoparticle reducing the total immunochemical activity of the resulting targeting nanogels. The immunochemical activity of anti-

VEGFR2 nanogels amounted to more than 70% of the initial activity of the free anti-VEGFR2 monoclonal antibody.

In order to investigate whether produced anti-VEGFR2 nanogels could selectively accumulate in C6 and U-87 MG cells (Fig. 3, A and B), immunohistochemical analysis was performed. It has been shown the possibility of anti-VEGFR2 nanogels bind with the antigen expressed on the cells surface. Nonmodified nanogels did not accumulate in cells (Fig. 3, C), whereas a weak fluorescent signal was detected in the case of nanogels conjugated with nonspecific antibodies (Fig. 3, D), which was due to nonspecific adhesion or sorption of the protein component of nanogels. Thus, specific binding of the anti-VEGFR2 monoclonal antibody with the antigen on the cells allowed nanogels selectively accumulate in glioma cells, as well as U-87 MG cells (Fig. 4) in contrast to the nonspecific and nonmodified nanogels.

Fig. 4. Fluorescence analysis of nanogel accumulation on U-87 MG cells. Cell visualization after treatment with anti-VEGFR2 nanogels. Nanogels (green), nuclei (blue).

CONCLUSIONS

The tumor vasculature is an attractive drug target, and efficient selective delivery of drugs are in demand. We have synthesized nanogels targeted to VEGFR2. This study provides findings that (i) produced anti-VEGFR2 antibodies might be used as a homing device, (ii) generated anti-VEGFR2 nanogels specifically bound to VEGFR2 presented on the brain tumor cells *in vitro*, but not control nanogels.

In summary, anti-VEGFR2 nanogels can be promising drug carriers for delivery therapeutics to tumor cells and vasculature and further studies need to evaluate their ability to deliver nanocarrier to the tumor tissue *in vivo*

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